JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1994, p. 2780–2785
0095-1137/94/$04.00+0

Vol. 32, No. 11

Transmission of *Rickettsia tsutsugamushi* Strains among Humans, Wild Rodents, and Trombiculid Mites in an Area of Japan in Which Tsutsugamushi Disease Is Newly Endemic

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Received 12 May 1994/Returned for modification 13 June 1994/Accepted 2 August 1994

Thirty-two newly isolated strains of *Rickettsia tsutsugamushi*, 14 from patients with tsutsugamushi disease, 12 from wild rodents, and 6 from trombiculid mites parasitizing rodents in Gifu Prefecture, Japan, were examined for reactivities to 12 monoclonal antibodies by an indirect fluorescent-antibody test to classify their antigenicities. All of the isolates could be classified into one of six groups (KN-1, KN-2, KN-3, GJ-1, R158, and R161) according to their reactivities to the monoclonal antibodies. The KN-1 and GJ-1 strains that are prevalent among patients from Gifu Prefecture had the same reactivities as the Kawasaki and Kuroki strains, respectively, which have been isolated and are prevalent in the Miyazaki and Kagoshima prefectures in southwest Japan. The isolates from patients were different in serotype from those from rodents and mites (*Leptotrombidium pallidum*). The KN-2 and KN-3 strains were most prevalent among patients and among rodents and mites, respectively. No close similarity between KN-2 and other strains tested was observed. KN-3 is only a minor contributor to diseases in patients in Gifu Prefecture; however, it was proven that the same strain was prevalent in Niigata Prefecture in northern Japan. Thus, Gifu Prefecture is an area where southern, northern, and local strains are found. We hypothesize that humans are prone to infection with KN-2, GJ-1 (very similar to Kuroki), and KN-1 (very similar to Kawasaki), probably by infestation with *Leptotrombidium scutellare*. While both *L. scutellare* and *L. pallidum* parasitize wild rodents and may carry any rickettsial strain, the most virulent strain, KN-3, is predominant among wild rodents. Antigenic analysis using monoclonal antibodies to *R. tsutsugamushi* should be useful for epidemiological studies of infection with this organism.

The number of patients with tsutsugamushi disease in Japan increased for about 10 years until 1984, after which it leveled off. The reasons for this change in prevalence are obscure. We have investigated the epidemiology of the disease in Gifu Prefecture (3–6), where no cases were reported until 1982, though since then the number of patients in the district has been ranked among the highest among prefectures in which tsutsugamushi disease is endemic.

Rickettsia tsutsugamushi, the causative agent of tsutsugamushi disease, has antigenic variants such as the Karp, Gilliam, and Kato strains. These three strains have been used as a set of standard strains for the diagnosis and analysis of the antigenicity of isolates. However, many isolated strains which are distinguishable from the prototype strains in antigenicity or virulency to mice have been identified by several investigators both in Japan (9, 14–16, 18) and in Korea (1), where the number of patients has also rapidly increased in recent years. The host range of *R. tsutsugamushi* is broad. Several species of mites and many species of rodents are infected in nature (11). We have isolated *R. tsutsugamushi* from wild rodents (*Apodemus speciosus*) captured in several areas in Gifu Prefecture (3–6). The host mites of *R. tsutsugamushi* have also been investigated. *Leptotrombidium pallidum* and *Leptotrombidium scutellare* have been shown to transmit rickettsiae, and a few other species are believed to carry rickettsiae in some local areas in Japan (13). We have noted a discrepancy in that we have easily isolated Karp-like rickettsiae from both *L. pallidum* and wild rodents, even though most patients in this prefecture have displayed anti-Gilliam-strain antibody responses. Therefore, it is very important to clarify the transmission of each rickettsial strain.

Diagnosis of the antigenic variation of *R. tsutsugamushi* would be useful for the epidemiological study of the transmission of the disease and for speculation about virulencies of rickettsial strains to humans. In this study, we analyzed the antigenicities of *R. tsutsugamushi* strains isolated from patients, wild rodents, and trombiculid mites by using monoclonal antibodies (MAbs); in this paper, we elucidate the endemic strains in the three host organisms and hypothesize about the transmission of each rickettsial strain.

MATERIALS AND METHODS

Rickettsiae. The Karp, Gilliam, and Kato strains of *R. tsutsugamushi* were supplied by the Toyama Prefectural Institute of Public Health, Toyama, Japan. The Kawasaki, Kuroki, and 423H-2 strains were kindly provided by A. Tamura, Niigata College of Pharmacy, Niigata, Japan. These six strains were propagated in L cells.

Isolation of *R. tsutsugamushi*. To isolate local strains of *R. tsutsugamushi*, blood samples from patients, spleen homogenates from wild rodents (*A. speciosus*), and whole-body homogenates of larval mites (*L. pallidum*) were injected intraperitoneally into ddY mice, as previously described (2). Briefly, blood samples were collected from patients who had high fevers and skin eruptions. Wild rodents were captured with rodent traps at several places in areas where *R. tsutsugamushi* is endemic. After splenectomy, the rodents were sacrificed, and

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TABLE 1. Antibody responses among patients and wild rodents to standard strains of *R. tsutsugamushi*

Serum source (*n*)	% of patients or rodents with antibody response[a]						
	Kp=Gl=Kt	Kp>others	Gl>others	Kt>others	Kp=Gl>Kt	Kp=Kt>Gl	Gl=Kt>Kp
Patients (109)	7.3	6.4	62.4	5.5	2.8	7.3	8.3
Wild rodents (64)	4.7	57.8	4.7	10.9	1.6	18.7	1.6

[a] =, antibody titers were the same for indicated strains; >, antibody titer was higher for the indicated strain; Kp, anti-Karp-strain antibody titer; Gl, anti-Gilliam-strain antibody titer; Kt, anti-Kato-strain antibody titer.

chigger mites were collected from these animals. Ten to 14 days after injection, the spleens were harvested from the mice, homogenized, and injected into new mice. After two or three passages, the spleens and sera were harvested from symptomatic mice for cell adaptation and serotyping, respectively. Weakly virulent strains provoking only splenomegaly in haired mice were further passaged in ICI nude mice. Virulencies were further estimated by infecting three or four BALB/c mice with 1:10 (wt/vol) spleen homogenate from each infected mouse. The strains which killed no mice were judged nonlethal, whereas the others (the lethal strains) killed all mice. The isolated strains were propagated in BS-C-1 cells (for nonlethal strains) or L cells (for lethal strains) to make the antigen for the indirect fluorescent-antibody assay (IFA).

MAbs. The details for five cross-reactive MAbs (Kp/1F11, Kp/C6, Kt/3B2, Kp/1C10, and Kt/3C2), Karp-specific Kp/D11, Kato-specific Kt/1D2 and Kt/2D9, and Gilliam-specific Gi/E4 were described previously (18), as were those for the Gilliam-specific MAb GM165 (1). The details of the production of MAbs were essentially the same as those described previously (18). Briefly, BALB/c mice were infected with each of the prototype strains and then given an intravenous booster challenge. Hybridomas were prepared by fusing the immunized spleen cells and NS-1 myeloma cells by using polyethylene glycol. Hybridomas secreting antirickettsial antibodies were cloned and injected into pristane-primed BALB/c mice to produce ascitic fluid.

IFA. *R. tsutsugamushi* strains propagated in BS-C-1 or L cells were collected when the complete cytopathic effect appeared, washed with phosphate-buffered saline (PBS), and then spotted on slide glass, dried, fixed with acetone, and used for IFA antigens. For human serum, serum from *A. speciosus*, and mouse serum and/or ascitic fluid, fluorescein isothiocyanate-labelled anti-human immunoglobulin G (heavy and light chain), anti-rat immunoglobulin G (heavy and light chain), and affinity-purified anti-mouse immunoglobulin G (heavy and light chain) goat sera (Cappel Company, Malvern, Pa.), respectively, were used. Sample sera and MAbs (ascitic fluid) were diluted with PBS from 1:20 by doubling dilution and identified by the IFA. The maximum dilution of the serum or the MAb which revealed rickettsia-specific fluorescence was considered the titer.

RESULTS

***R. tsutsugamushi* isolated in Gifu Prefecture.** We tested sera from 109 patients and positive sera from 64 of 156 wild rodents. A Gilliam-like antibody response was most common among patients, while a Karp-like response was most common among wild rodents (Table 1). Furthermore, we isolated 14 rickettsial strains from patients, 40 from wild rodents, and 6 from trombiculid mites (*L. pallidum*). Antibody responses among BALB/c mice infected with these isolates confirmed the pattern in Table 1 (Table 2). Also, strains isolated from *L. pallidum* appeared to be similar to strains prevalent among wild rodents. All strains from trombiculid mites, all but three strains from rodents, and one strain from a patient were lethal to BALB/c mice. All 20 isolates from both patients and mites and 12 of 40 isolates from rodents were inoculated into cells to investigate their antigenicities. Twelve isolates from rodents, including three nonlethal ones, representing different types of antibody responses in hosts (Table 2) and different geographic areas (Fig. 1) were selected for investigation.

Antigenic analysis of isolates with the MAbs. Three hybridomas (Kp/2-3, Kp/13-5, and Gi/2E3) that secreted strain-specific MAbs to standard strains of *R. tsutsugamushi* were newly established and added to the reagents for analysis. The reactivity of each MAb is shown in Table 3. We had previously reported that a Karp-specific MAb, Kp/D11, reacted with the isolated strains KN-1, -2, -3, and -4 (18). However, we found that there were nonspecific reactivities which resulted from using this MAb with nonpurified fluorescein isothiocyanate-labelled anti-mouse immunoglobulin G. MAb Kp/D11 was finally shown to react with only the Karp and R161 strains in the present analysis by using affinity-purified fluorescein isothiocyanate-labelled antibody. Finally, each isolate could be classified into one of six groups (KN-1, KN-2, KN-3, GJ-1, R158, and R161) according to their reactivities with eight strain-specific and five cross-reactive MAbs to standard strains (Table 3). The KN-1 group did not react with the eight

TABLE 2. Antibody responses of BALB/c mice inoculated with *R. tsutsugamushi* strains isolated from patients, wild rodents, and trombiculid mites

Isolate source (*n*)	% of BALB/c mice with antibody response[a]						
	Kp=Gl=Kt	Kp>others	Gl>others	Kt>others	Kp=Gl>Kt	Kp=Kt>Gl	Gl=Kt>Kp
Patients (14)	0	28.6	64.3	0	0	7.1	0
Wild rodents (40)	2.5	75.0	2.5[b]	2.5	0	15.0	2.5
Trombiculid mites (6)	0	66.7	0	0	0	33.3	0

[a] =, antibody titers were the same for indicated strains; >, antibody titer was higher for the indicated strain; Kp, anti-Karp-strain antibody titer; Gl, anti-Gilliam-strain antibody titer; Kt, anti-Kato-strain antibody titer.

[b] Only a very weak response was seen to the Gilliam strain.

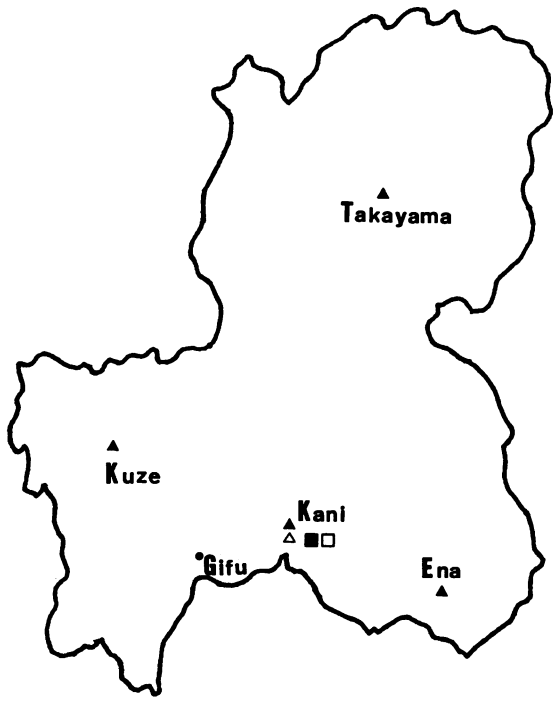

FIG. 1. Distribution of rickettsial strains among wild rodents in Gifu Prefecture. ▲, KN-3; ■, KN-1 (also called Kawasaki); □, R158; △, R161.

strain-specific MAbs and reacted with the cross-reactive MAbs Kp/1F11, Kp/C6, and Kt/3B2. The KN-2 group reacted with the Gilliam-specific MAb GM165 and with the cross-reactive MAbs Kp/1F11, Kp/C6, Kt/3B2, and Kp/1C10. However, the KN-2 group differed from the Gilliam strain in its reactivity with the Gilliam-specific MAbs Gi/E4 and Gi/2E3 and with the cross-reactive MAb Kp/1F11. The KN-3 group reacted with the Karp-specific MAb Kp/2-3 and with five cross-reactive MAbs. The KN-3 group was clearly distinguished from the Karp strain by its reactivity with the Karp-specific MAbs Kp/D11 and Kp/13-5. The R161 group was more similar to Karp; however, it either did not react with the cross-reactive MAbs Kp/C6 and Kt/3B2 or its reactivities were extremely low. The GJ-1 group reacted with the Karp-specific MAb Kp/13-5 and with five cross-reactive MAbs. However, the GJ-1 group differed from the Karp strain in its reactivity with the Karp-specific MAbs Kp/D11 and Kp/2-3. The R158 group did not react with the eight strain-specific MAbs but did react with the cross-reactive MAbs Kp/1F11, Kp/C6, Kt/3B2, and Kp/1C10. The R158 group differed from the KN-1 and KN-2 groups in its reactivities with the cross-reactive MAb Kp/1C10 and the Gilliam-specific MAb GM165, respectively (Table 3). All the isolates from patients which displayed Gilliam-type antibody responses in Table 2 were classified as members of the KN-1 or KN-2 group, while those showing Karp-type responses were classified as members of the GJ-1 group. The antibody response in humans in which the titers of antibodies to Karp and Kato were equal (i.e., a Karp=Kato type of antibody response) indicated a KN-3 infection. All the isolates from trombiculid mites were proven to be KN-3. The isolates from rodents which displayed both Karp-type and Karp=Kato-type responses were identified as KN-3. Nonlethal strains isolated from rodents were identified as KN-1 (Gilliam=Kato type) and R158 (Kato type and Karp=Gilliam=Kato type). A Gilliam-type response indicated an R161 infection. Of all the isolates, KN-3 and R161 were lethal to BALB/c mice (Table 4).

Geographical distribution of serotypes. KN-3 has been isolated from wild rodents throughout the Gifu Prefecture, including the Ena area, where no human cases have been reported (Fig. 1). Other isolates from rodents not tested here are also suggested to be KN-3 because of their antibody responses (Table 1) and high virulencies. The origin of KN-3 has been identified as *L. pallidum* (Table 4). KN-3 (also called 423H-2) was also isolated from a patient in Gifu Prefecture and from another in Niigata Prefecture (Table 5; Fig. 2). While the dominant strains among patients in the Gifu area were KN-2 and GJ-1 (Table 4), the latter was proven to be the same as the Kuroki strain in a previous study (17). KN-1, which was demonstrated to be the same as the Kawasaki strain (17), and GJ-1 (also called Kuroki) were the dominant strains in the Kagoshima-Miyazaki area and have also been detected in Shizuoka, Kanagawa, and Tokyo (Fig. 2).

TABLE 3. Reactivities of standard strain-specific and cross-reactive MAbs with isolates of *R. tsutsugamushi*

Clone	IFA titer to[a]:								
	Karp	Kato	Gilliam	R161	KN-3	GJ-1	KN-2	R158	KN-1
Kp/D11	640	—[b]	—	2,560	—	—	—	—	—
Kp/2-3	20,480	—	—	2,560	5,120	—	—	—	—
Kp/13-5	10,240	—	—	2,560	—	5,120	—	—	—
Kt/1D2	—	20,480	—	—	—	—	—	—	—
Kt/2D9	—	1,280	—	—	—	—	—	—	—
Gi/E4	—	—	10,240	—	—	—	—	—	—
Gi/2E3	—	—	2,560	—	—	—	—	—	—
GM165	—	—	2,560	—	—	—	2,560	—	—
Kp/1F11	2,560	80	—	80	80	2,560	2,560	2,560	2,560
Kp/C6	1,280	1,280	1,280	—	40	1,280	1,280	1,280	640
Kt/3B2	2,560	2,560	2,560	20	160	1,280	2,560	2,560	640
Kp/1C10	1,280	80	80	2,560	1,280	1,280	640	160	—
Kt/3C2	2,560	2,560	—	320	1,280	1,280	—	—	—

[a] Titers are expressed as reciprocals of the highest dilution of immune ascitic fluid causing rickettsial fluorescence.
[b] —, less than 1:20.

TABLE 4. Number of rickettsial strains isolated from patients, wild rodents, and trombiculid mites

Group[a]	No. of strains (pattern of Ab response[b]) isolated from[c]:		
	Patients	Rodents	Mites[d]
KN-1 (NL)	2 (Gl)	1 (Gl=Kt)	0
KN-2 (NL)	7 (Gl)	0	0
GJ-1 (NL)	4 (Kp)	0	0
R158 (NL)	0	2 (Kt, Kp=Kt=Gl)	0
KN-3 (L)	1 (Kp=Kt)	8 (Kp, Kp=Kt)	6 (Kp, Kp=Kt)
R161 (L)	0	1 (Gl)	0

[a] NL, nonlethal; L, lethal.
[b] Pattern of antibody (Ab) responses in BALB/c mice inoculated with each strain (see Table 2). =, antibody titers were the same for indicated strains; >, antibody titer was higher for the indicated strain; Kp, anti-Karp-strain antibody titer; Gl, anti-Gilliam-strain antibody titer; Kt, anti-Kato-strain antibody titer; Gl, Gl>others; Kp=Kt, Kp=Kt>Gl.
[c] For patients, rodents, and mites, n = 14, 12, and 6, respectively.
[d] *L. pallidum.*

DISCUSSION

Among the isolates of *R. tsutsugamushi* used in this study, variations were recognized in the reactivities to the MAbs against standard strains. We could divide the 32 isolates from patients, wild rodents, and trombiculid mites in Gifu Prefecture into six serogroups, i.e., KN-1, KN-2, KN-3, GJ-1, R158, and R161, by using type-specific and cross-reactive MAbs against standard strains. These MAbs were also used to differentiate the isolates in other areas of Japan. As mentioned earlier KN-1 and GJ-1 are very similar to the Kawasaki and Kuroki strains, respectively (17). The Kagoshima-Miyazaki and Gifu-Shizuoka areas are located on different islands, 600 km apart. It is mysterious that no Kawasaki or Kuroki strain has so far been reported between these two areas. We compared the antigenicities of the Japanese strains isolated in the Gifu area with the antigenicity of the Boryoung strain, which was the most prevalent serotype in Korea. There are no cross-reactivities between the Boryoung and any of the Japanese strains except for GJ-1 (unpublished data).

The KN-2 group, reactive with the Gilliam-specific MAb GM165, had more antigenic similarities to the Gilliam strain than to the other isolated strains. However, it has been demonstrated that KN-2 differs from all strains, including Gilliam, yet no similar strain has been found among strains in Japan, Korea, and Thailand (unpublished data). KN-2 may thus be a local variant strain of rickettsia.

The KN-3 group reacted with the Karp-specific MAb Kp/2-3, suggesting that KN-3 is related to the Karp strain, though our analysis clearly distinguished KN-3 from Karp (Table 3). Furthermore, no Korean or Thai strains were very similar to KN-3. It was found in this study that KN-3 is very similar to the 423H-2 strain isolated from a patient in Niigata Prefecture (Table 4). Many Karp and/or Karp-like infections have been reported in the area between Gifu and Niigata and in the northern part of the main island of Japan (Honshu), and we predict that more KN-3 infections would be found if MAbs which differentiated KN-3 from Karp were used. Recently, we have demonstrated that KN-3 is also endemic in Tokushima (Fig. 2), which is located on another island (unpublished data).

Vertical transmissions of *R. tsutsugamushi* in trombiculid mites would be the only mechanism of its maintenance in nature, as described by G. Rapmund (10). So, the serotypes of rickettsiae in the species or even in the colonies of the same species of the vector mites would be different. *Leptotrombidium akamushi*, *L. pallidum*, and *L. scutellare* have been known as vector mites in Japan (13). The distribution of *L. akamushi* organisms which transmit Kato and/or Kato-like strains has been limited to the northern part of Honshu; moreover, the area in which *L. akamushi* is endemic has been diminishing. No *L. akamushi* organisms have been detected in the Gifu area (2). We have already demonstrated that *L. pallidum* carries a Karp-like rickettsia (2). This Karp-like strain, however, has been identified as the KN-3 strain by the analysis with MAbs used in this study. Strain KN-3 has thus far been isolated from only one patient in Gifu Prefecture (17). Moreover, the majority of wild rodents appear to be infected with the KN-3 strain, which displays a Karp-like or Karp=Kato type of antibody response among wild rodents (Tables 1 and 2; Fig. 1).

In the northern part of Japan, including the northern part of Gifu Prefecture, *L. pallidum* appears in early winter and remains under snow until spring (12). Therefore, more cases of infections in humans are reported in both early winter and spring, when people are in contact with the soil as they collect wild potatoes, mushrooms, plants, and fruits, whereas both *L. pallidum* and *L. scutellare* appear only in early winter in southern Japan, including the survey areas located in southwest Gifu Prefecture (2). Therefore, it is concluded that cases reported in the spring result from the appearance of *L. pallidum* parasitized with a KN-3 strain. Fatal cases of infection in this prefecture occurred only in the spring. Also, KN-3 is the only strain deadly to mice detected in this area, except for a minor variant, or R161.

In our previous study, it was demonstrated that *L. scutellare* was the most predominant strain and *L. pallidum* was the second most predominant strain found in early winter at the south or southwest survey points. A further correlation between the appearance of *L. scutellare* and cases of human disease suggested to us that the majority of cases in early winter resulted from the transmission of rickettsiae by *L. scutellare* (2). However, we had failed to isolate any rickettsiae from *L. scutellare* (3). We formerly concluded that the rickettsia possession rate of *L. scutellare* was too low (less than 0.1%) (3) to isolate rickettsiae. But it is reasonable to assume that there should be small areas where the populations of rickettsia-possessing *L. scutellare* are highly dense. Actually, a Ka-

TABLE 5. Close correlation between KN-3, isolated in Gifu Prefecture, and 423H-2, isolated in Niigata Prefecture

Clone	IFA titer to[a]:	
	KN-3	423H-2
Kp/D11	—[b]	—
Kp/2-3	5,120	2,560
Kp/13-5	—	—
Kt/1D2	—	—
Kt/2D9	—	—
Gi/E4	—	—
Gi/2E3	—	—
GM165	—	—
Kp/1F11	80	160
Kp/C6	40	—
Kt/3B2	160	160
Kp/1C10	1,280	1,280
Kt/3C2	1,280	640

[a] Titers are expressed as reciprocals of the highest dilution of immune ascitic fluid causing rickettsial fluorescence.
[b] —, less than 1:20.

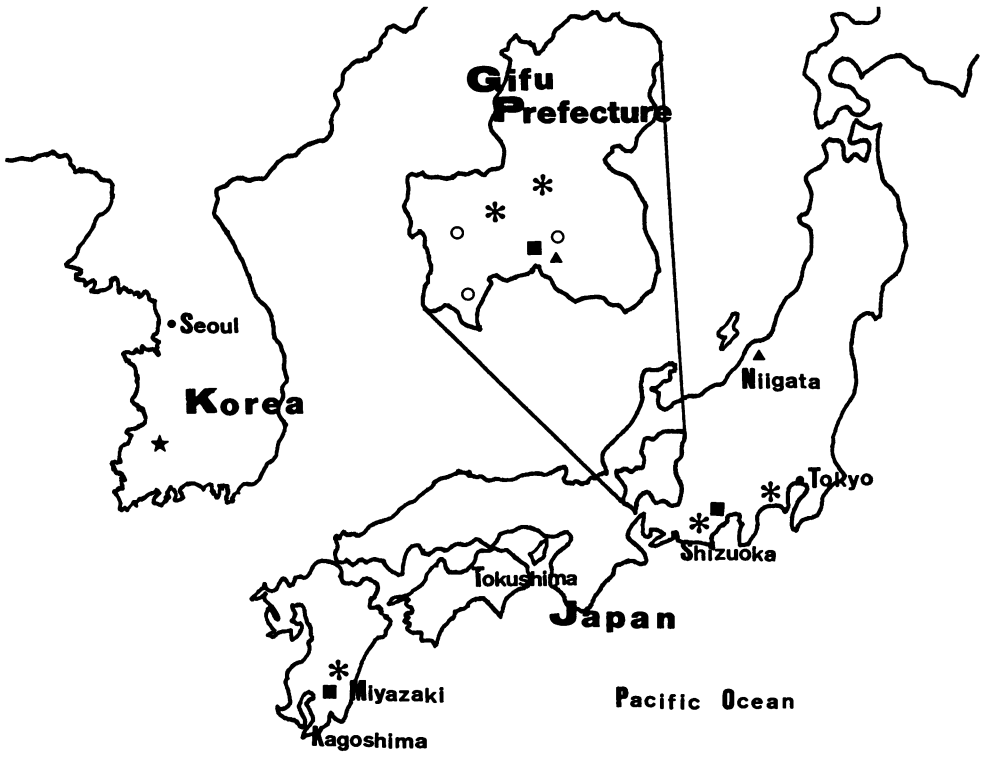

FIG. 2. Geographical distribution of rickettsial strains among patients in Japan and Korea. *, GJ-1 (also called Kuroki); ■, KN-1 (also called Kawasaki); ○, KN-2; ▲, KN-3 (also called 423H-2); ★, Boryoung (similar to Kuroki).

wasaki-type strain was isolated from *L. scutellare* (7). Moreover, *L. scutellare* has seldom been found where Kawasaki or Kuroki was not endemic.

Finally, we hypothesize that the transmission of each rickettsial strain occurs via two species of trombiculid mite. Humans are prone to infection with KN-1, KN-2, and GJ-1, probably via *L. scutellare*, which is likely to parasitize humans, even though *L. pallidum* and *L. scutellare* are equally prevalent. Also, dermatitis caused by *L. scutellare* strains which do not possess any rickettsiae is common in areas in which rickettsiae are endemic. On the other hand, wild rodents have a chance to be infected with rickettsial strains from both *L. scutellare* and *L. pallidum*, which parasitize wild rodents equally (2). However, weakly virulent strains may be masked by highly virulent KN-3 strains or lost during isolation procedures involving passaging in mice. This hypothesis is supported by the fact that 3 strains (of 40) of weakly virulent rickettsiae were isolated from wild rodents. These rodents appeared to be infected with a weakly virulent rickettsial strain by *L. scutellare* before being infected with KN-3 from *L. pallidum.*

This hypothesis may explain the high endemicity of the disease in Gifu Prefecture. However, a sudden increase in the number of patients requires another explanation. We have no evidence that the number of trombiculid mites is on the increase. The increasing number of patients may well reflect increasing knowledge about this infectious disease and an increase in the use of serodiagnosis. The majority of patients have, in the past, been treated easily with chloramphenicol or tetracycline before being diagnosed. Prior to the antibiotic age, endemic typhus was present in many areas in Japan, although it was nonlethal in Gifu Prefecture (8) and may therefore have been overlooked.

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